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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 5 July 2002 with an application for Letters Patent number 520019 made by Canterprise Limited

I further certify that pursuant to a claim under Section 24(1) of the Patents Act 1953, a direction was given that the application proceed in the name of SYFT Technologies Limited.

Dated 23 July 2003.

PRIORITY DOCUMENT
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Neville Harris
Commissioner of Patents

Patents Form No. 4

PATENTS ACT 1953

PROVISIONAL SPECIFICATION

A method of assaying the antioxidant activity of pure compounds, extracts and biological fluids

We, Canterprise Limited, a New Zealand company, of Forestry School Building, Forestry Road, Ilam, Christchurch, New Zealand, do hereby declare this invention to be described in the following statement:

TECHNICAL FIELD

The present invention relates to a novel method of ethylene analysis for assaying the antioxidant activity of pure compounds, extracts and biological fluids using Selective Ion Flow Tube Mass Spectrometry (SIFT-MS). The assay provides an in vitro method for the sensitive, rapid and continuous, real time, absolute concentration determination and quantification of oxidative radical activity of pure compounds, extracts and biological fluids as well as the antioxidant activity of pure compounds, extracts and biological fluids.

BACKGROUND ART

Formation of reactive oxygen species in aerobic organisms is an unavoidable consequence of the coupling of oxidative phosphorylation of ADP with the reduction of molecular oxygen by four electrons to water. Other sources of oxidative radicals include microsomal and photosynthetic electron transport chains, active phagocytosis, and the activity of a variety of enzymes that produce different reactive species as intermediates.

The in vivo generation of oxidative free radicals results in the chemical degradation of cellular organelles, membranes, deoxyribosenucleic acids (DNA), and other structural elements as well as the disruption of biochemical pathways, transduction and translation events as well as genetic replication and repair. These effects may be translated into tissue, and organ damage and malfunction leading to a wide variety of diseases and the generation of malignancies. These damaging changes may be produced in various tissues by trauma, environmental hazards, metabolic defects, inflammation or infections as well as the natural responses to cellular aging, or natural and acquired immunity to foods, commensal micro-organisms, environmental agents or surveillance against spontaneously occurring tumourogenesis. Natural or synthetic dietary or parenterally administered agents capable of reducing or eliminating oxidative free radicals in cells and tissues are currently thought to protect against actual or potential oxidative damage in vivo.

The measurement of free radical generation and oxidation as well as oxidative radical scavenging by naturally occurring or extraneous molecules is currently both complex and time consuming. This invention provides a rapid, continuous, sensitive means of measuring oxidative free radical and scavenging activities without calibration, standards or complex sample

ration using SIFT-MS. Its applications extend from measuring the biochemical production of oxidative free radicals in vivo or in vitro to the capacity of oxidative radical scavengers to impede, inhibit or compete with the generation or activity of oxidative free radicals. This SIFT-MS analytical system can be used to calibrate and standardise other in vitro measurement techniques, monitor and quantify oxidative chemical generation and reactivity, monitor and quantify antioxidant generation and reactivity and determine the relative rates of the generation and the relative reactivities of those systems.

Cells have evolved oxidative defenses that involve specially adapted enzymes as well as membrane-associated and aqueous phase molecules. The production of reactive oxygen species in vivo does not necessarily imply cellular damage but oxidative stress is thought to occur when the production of those oxidative radicals exceeds the scavenging, protective capacity of the endogenous antioxidants.

In vitro assays of oxidative free radical activity based on the time required to obtain maximum oxygen consumption (Wayner et al. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. FEBS Lett. 1985;187;33-37), phycoerythrin emission fluorescence (Glazer AN. Fluorescence-based assay for reactive oxygen species: A protective role for carnitine. FASEB J. 1988;2:2487-91) and peroxyl radical oxidation of α –keto-γ-methiolbutyric acid (KMBA) to ethylene by gas chromatography (Winston GW, et al. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. Free Radical Biology & Medicine 1998;24:480-93) have been described.

The present method is based on the known partial inhibition of ethylene formation in the presence of antioxidants that compete with KMBA for oxyradicals. This has been measured previously in the headspace of a reaction vessel by gas chromatography to derive the Total Oxyradical Scavenging Capacity Assay (TOSCA).

OBJECT OF THE INVENTION

An object of the present invention is to measure the concentration of ethylene as an assay for antioxidant activity using the SIFT-MS technique.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

The method briefly stated:

Peroxyl radicals were generated by the thermal homolysis of AAPH (2,2'-Azobis(2-amidinopropane)dihydrochloride) at 35°C and at temperatures between 35°C-39°C.

2. An assay was carried out using 0.2mM of substrate KMBA and 20mM of AAPH in 100mM of phosphate buffer at pH 7.4

3. The TOSC values were measured for different concentrations of antioxidants, trolox, ascorbic acid, glutathione, uric acid and Pinus radiata bark extract Enzogenol® and human plasma

4. Quantification of total oxyradical scavenging capacity = 100-(∫ SA/ ∫ CAx100)
∫ SA = integrated areas of the sample curve
∫ CA = integrated areas of the control curve

Selected Ion Flow Tube Mass Spectrometry

Modifications of the SIFT-MS technique for analyzing trace components of gas mixtures have been described by Milligan DB, Wilson PF, Mautner MN, Freeman CG, McEwan MJ, Clough TJ, Sherlock RR. Real-Time, High-Resolution Quantitative Measurement of Multiple Soil Gas Emissions: Selected Ion Flow Tube Mass Spectrometry. J. Environ. Qual. 2002 31: 515-524. SIFT-MS measures trace gases in complex mixtures such as air, breath and the headspace above liquids, allowing the analysis of a single exhalation of breath in real time, giving immediate results without the need for pre-concentration of the volatile gas compounds or calibration using standards.

SIFT-MS utilizes selective chemical ionization, using precursor ions generated by electron impact, by microwave discharge or by glow discharge. The precursor ions are mass selected using a quadrupole mass filter to inject mass selected precursor ions into a stream of helium carrier gas and allowed to reach thermal equilibrium. Positive or negative precursor ions may be chosen. The precursor ion must be unreactive with the bulk gas within which the trace species is carried, but react rapidly with the trace species of interest. O_2^+ ions are used to measure ethylene in this assay. The reaction vessel headspace sample is introduced into the carrier gas stream at a calibrated rate via a heated capillary inlet, alternatively a mass flow controller or calibrated leak valve could be used. Following this, the trace components within the sample gas mixture undergo reaction with the precursor ions in the helium bath gas. The concentration of a (or each) trace species VOC in the gas mixture is then calculated from the observed number densities of the precursor and product ions as measured by a second mass filter (quadrupole or time-of-flight mass spectrometer) in conjunction with a particle multiplier and specialized software interface, library and data processor/analyser. In order to calculate the actual partial pressure of the trace

es it is essential to know the rate of and products formed by the reaction of the precursor ion with the trace neutral under the conditions within the flow tube.

. This SIFT-MS analysis of ethylene is performed in real time, with no sample preparation, no calibration and without standards. In one preferred embodiment of the invention the assay provides an in-vitro method for the sensitive, rapid and continuous, real time, absolute concentration determination and quantification of oxidative radical activity of pure compounds, extracts and biological fluids as well as the antioxidant activity of pure compounds, extracts and biological fluids.

In the present invention the reaction between O_2^+ and ethylene that is measured is as follows.

$$O_2^+ + C_2H_4 \rightarrow C_2H_4^+ + O_2 \qquad k= 1.0e\text{-}9\ cm^3\ molecule^{-1}\ s^{-1}$$

The precursor and product ions are scanned over predetermined ranges of mass-to-charge ratio, m/z, for a given time. For this invention, the downstream analytical mass filter was switched between the m/z value of the precursor ion (m/z 32) and the m/z value of $C_2H_4^+$ (m/z 28) ethylene to target the chosen oxyradical/KMBA end product trace gas species. The partial pressure of ethylene in the sample is then calculated immediately, on line, from the precursor and product ion count rates. In this way, rapid changes in ethylene concentrations are monitored by SIFT-MS in sequentially obtained headspaces during, or at the end of the oxidative reaction.

The invention provides a SIFT-MS method for the determination, measurement and comparison of oxidative radical activity activity in vitro and in vivo in both natural and synthetic substances using any oxidant or antioxidant. The SIFT-MS method may be automated to determine and measure oxidative radical activity and antioxidant activity in any natural and/or synthetic substances.

The invention is referred to as the Total Oxyradical Scavenging Capacity – Selected Ion Flow Tube (TOSC-SIFT) assay and is a fast multi radical scavenging assay based on the partial inhibition of ethylene production by antioxidants.

BRIEF DESCRIPTION OF FIGURES

Fig.1 is a graph of the rate of reaction of KMBA oxidation by peroxyl radicals in the presence of increasing concentrations of trolox and ascorbic acid.

Fig. 2 is a graph of the rate of reaction of KMBA oxidation by peroxyl radicals in the presence of increasing concentrations of glutathione (GSH) and uric acid.

Fig. 3 is the regression of TOSC values of different antioxidants at different concentrations.

Fig. 4 is the reaction rate of KMBA oxidation by peroxy radicals in the presence of increasing amounts of human plasma.

Fig. 5 is the regression of TOSC values of human plasma at different concentrations.

BRIEF DESCRIPTION OF TABLES

Table 1 is the linear correlation between the different assays and sample concentration.

Table 2 is a comparison of relative total oxyradical scavenging capacity (TOSC) values (on a per unit concentration basis) of different antioxidants calculated from the TOSCA-SIFT-MS and other methods; TOSCA, ORAC, TRAP-1 (phycoerythrin) and TRAP-2 (oxygen electrode).

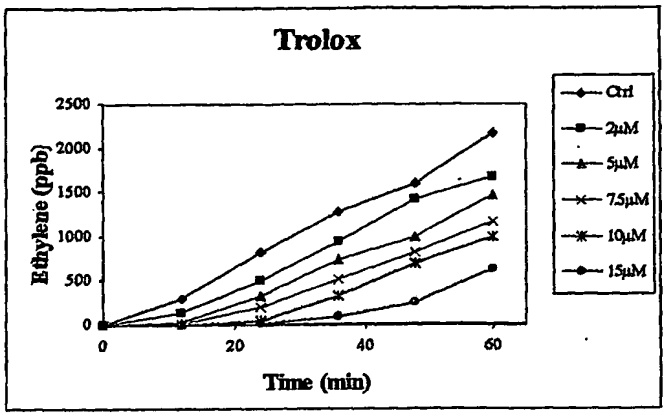
Table 3 is a comparison of relative total oxyradical scavenging capacity (TOSC) values (on a per unit weight basis) of different antioxidants calculated from the TOSCA-SIFT-MS and the other methods of TOSCA and ORAC.

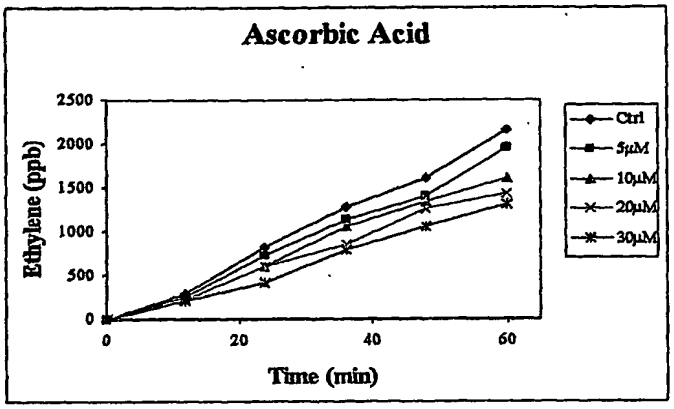
ANTAGES OF THE ASSAY METHOD

1. Fast sensitive method
2. No sampling needed and direct headspace analysis
3. The system is amenable to automation
4. Measures absolute concentrations
5. Ideal for serum and biological fluids
6. Choice of free radical generators
7. Choice of other oxidants (e.g., peroxynitrite and HOCl)

DESCRIPTION OF FIGURE 1

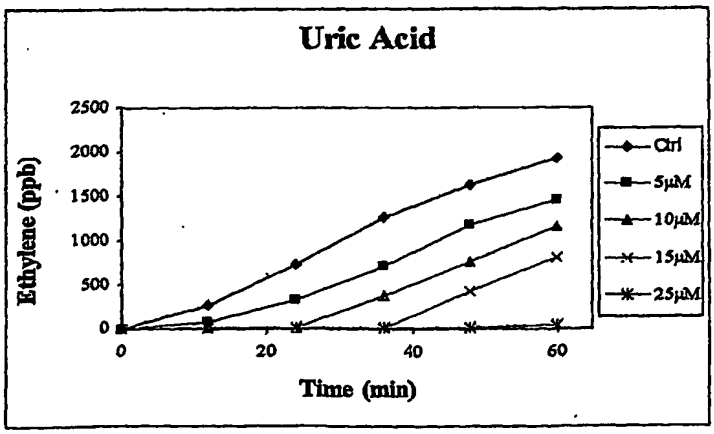
Rate of reaction of KMBA oxidation by peroxyl radicals in the presence of increasing concentrations of trolox and ascorbic acid

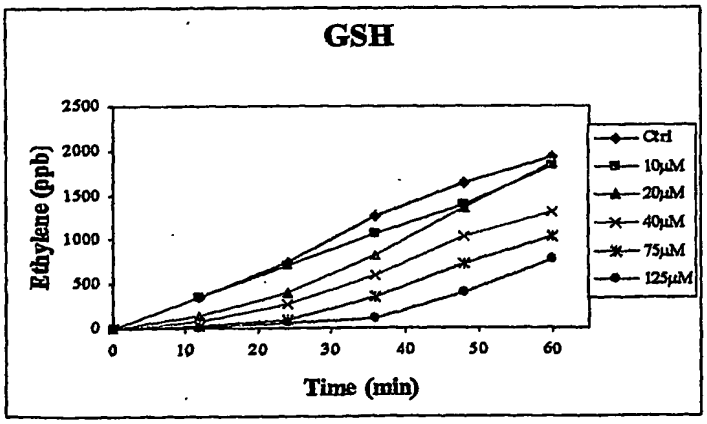




DESCRIPTION OF FIGURE 2

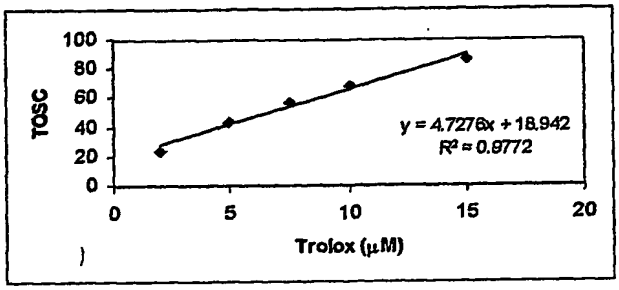
Rate of reaction of KMBA oxidation by peroxyl radicals in the presence of increasing concentrations of glutathione (GSH) and uric acid

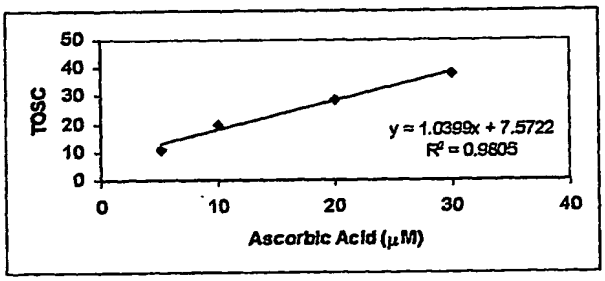


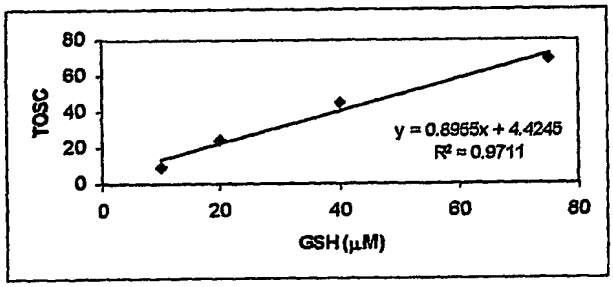


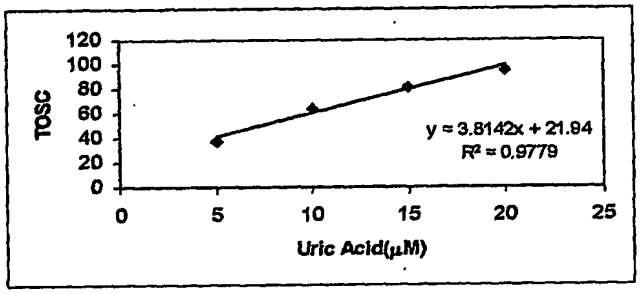
DESCRIPTION OF FIGURE 3

Regression of TOSC values of different antioxidants at different concentrations









DESCRIPTION OF TABLE 1

Linear correlation between the different assays and sample concentration

	TOSCA-SIFT	TOSCA-GC	ORAC
Trolox (uM)	2-15	2-20	0-3
Uric Acid (uM)	5-20	2-25	0-4
Ascorbic Acid (uM)	5-30	5-50	0-2
GSH (uM)	10-75	10-75	----

SIFT = selected ion flow tube-mass spectrometry

GC = gas chromatography (Winston et al 1998)

ORAC = oxygen radical absorbance capacity (Cao G, Alessio HM, Cutler RG. Oxygen radical absorbance capacity assay for antioxidants. Free Radic. Biol Med. 1993;14:303-11)

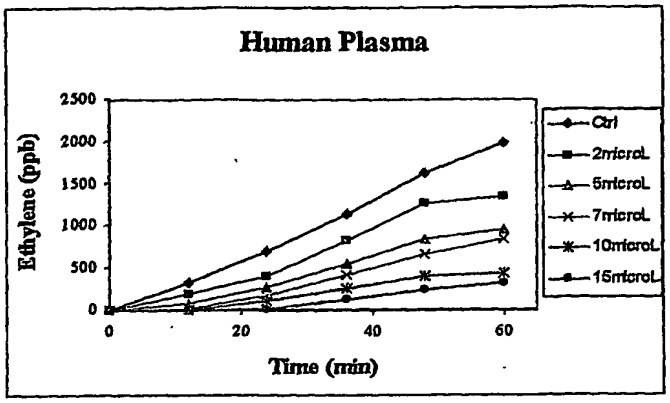
DESCRIPTION OF TABLE 2

A comparison of relative total oxyradical scavenging capacity (TOSC) values (on a per unit concentration basis) of different antioxidants calculated from the TOSCA-SIFT-MS and other methods; TOSCA, ORAC, TRAP-1 (phycoerythrin) and TRAP-2 (oxygen electrode)

	SIFT-MS	TOSCA	ORAC	TRAP-1	TRAP-2
Trolox	1	1	1	1	1
Ascorbic acid	0.36	0.46	0.52	0.75	0.85
GSH	0.22	0.19	—	—	0.18
Uric acid	1.08	0.70	0.92	0.85	0.65

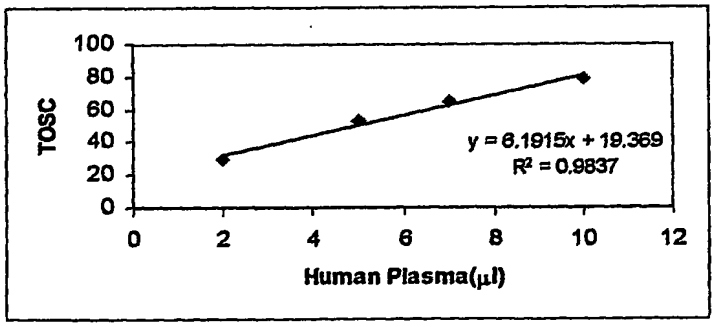
CRIPTION OF FIGURE 4

Kinetics of KMBA oxidation by peroxyl radicals in the presence of increasing concentration of human plasma



DESCRIPTION OF FIGURE 5

Regression of TOSC values of human plasma at different concentrations



[redacted]CRIPTION OF TABLE 3

A comparison of relative total oxyradical scavenging capacity (TOSC) values (on a per unit weight basis) of different antioxidants calculated from the TOSCA-SIFT-MS and the other methods of TOSCA and ORAC

	SIFT-MS	TOSCA	ORAC
Trolox	**1**	**1**	**1**
Ascorbic acid	**0.36**	**0.69**	**0.67**
GSH	**0.19**	**0.16**	**---**
Uric acid	**1.18**	**0.95**	**1.44**

Having described preferred embodiments of the invention it will be apparent to those skilled in the art that various changes and alterations can be made to the embodiments and yet still come within the general concept of the invention. All such changes and alterations are intended to be included in the scope of this specification.